

Tumor Promoters in Commercial Indoor-Plant Cultivars of the *Euphorbiaceae*

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Certain decorative indoor-plant cultivars are derived from toxic wild plant species. Native members of the *Euphorbiaceae* (spurge) contain highly irritating and tumor-promoting diterpene esters. Plant breeders and gardeners are constantly searching for less toxic cultivars of the popular *Euphorbiaceae* indoor plants. In this investigation, 22 commercial cultivars of *Euphorbiaceae* indoor plants were examined for tumor promoter contents by high-performance liquid chromatography (HPLC). Cultivars of *E. milii* (*E. lomii* hybrids), and in particular *E. leuconeura*, contained ingenol derivatives, whereas cultivars of *E. pulcherrima* and *Codiaeum variegatum* were devoid of these compounds. Tumor-promoting activity was assessed by induction of a luciferase reporter gene, which was placed under the control of an Epstein-Barr virus early antigen promoter. The response was closely correlated with ingenol ester content; the latex of the two *E. leuconeura* cultivars tested gave the strongest response. The HPLC and bioassay methods used in this study provide a basis for the development of nontoxic indoor-plant cultivars and perhaps for consumer-oriented labeling. **Key words:** EBV induction, *Euphorbiaceae*, indoor plants, ingenol derivatives, phorbol derivatives, tumor promotion. *Environ Health Perspect* 107:753–756 (1999). [Online 5 August 1999]

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Indoor plants are popular because of their beauty and their ability to improve room air quality. Oxygen is generated in rooms where plants are grown, and carbon dioxide particulate matter and air pollutants are removed or their concentrations decreased (1). A complete detoxification of formaldehyde has also been demonstrated (2–4). However, certain indoor-plant species may still contain toxic or irritating substances that are known products of plant secondary defense metabolism in the parent wild plant species. It is a continual effort of plant breeders to produce cultivars in which these metabolites are reduced or absent. These efforts are important because of cases where children or pets were poisoned by the consumption of leaf material of ornamental plants (5). Florists and horticulturists handling indoor plants often suffer from contact dermatitis due to toxic plant compounds (6,7).

Several of the most popular indoor-plant species are members of the *Euphorbiaceae*. Numerous cultivars have been produced and are available in garden centers and supermarkets. The potential for skin irritation or even tumor-promoting activity has been recognized (5,6,8), but no thorough study of commercial *Euphorbiaceae* indoor-plant species is available to date. The *Euphorbiaceae* or spurge family includes approximately 8,000 native species in 300 genera that occur in tropical and temperate regions all over the world (9). Most of them contain a milky latex that in many cases is toxic to animals and produces contact dermatitis [reviewed by Frohne and Pfänder (5)]. A wide range of irritant and tumor-promoting diterpene

esters with tiglane, ingenane, and daphnane skeletons have been isolated (10). A biotest for tumor promotion on mouse skin was established (11,12). A more recent biotest for the analysis of tumor-promoting chemicals is based on the induction of the Epstein-Barr virus (EBV) in cultured cells. Many substances that promote tumors in the mouse skin assay also induce the lytic cycle of EBV. Phorbol esters from spurge plants are active in both experimental systems (13).

We investigated a total of 22 commercial cultivars of *Euphorbia lomii* hybrids, *Euphorbia pulcherrima* (*Poinsettia*), *Euphorbia leuconeura*, and *Codiaeum variegatum* for tumor-promoting diterpenoids using high-performance liquid chromatography (HPLC). In addition, the tumor-promoting potential of plant extracts was measured by an EBV induction assay that used the reporter gene luciferase under the control of an EBV early antigen promoter in Raji cells (14). Marked differences in tumor promoter contents among the tested commercial indoor-plant cultivars were discovered.

Materials and Methods

Cultivation of indoor plants and collection of latex. The following plant cultivars were used in our investigations: *E. lomii* hybrids (= *E. lophogona* × *E. milii*; cultivars: Bianca, Gabi, Mariella, Marathon, Rosemarie); *E. pulcherrima* (cultivars: Bonita, Flirt, Freedom, Maren, Nobelstar, Peterstar, Regina); *E. leuconeura* [cultivar 1, local propagation at the Institute of Biochemical Plant Pathology (Oberschleißheim, Germany); cultivar 2, purchased from the Uhlig Nursery, Kernen,

Germany], and *Co. variegatum* (cultivars: Batic Red, Batic Green, Icteton, Mara, Pinocchio, Scarletta, Tamara, Yellow Tip). The plants were kept in a greenhouse under typical indoor conditions: temperature, day/night 22°C/18°C and low light conditions ~ 1 klx. Plants were potted in commercially available soil (Fruhstorfer Einheitserde Type T; Lauterbach, Germany) or in hydroponic culture. Nutrients were supplied by a standard solution (Flory 9; Euflor, Munich, Germany). White latex was drained into tubes from scalpel incisions into the stem and the leaves of the test plants.

Extraction of diterpene esters. Approximately 0.5 mL latex samples were weighed and extracted at once with 1 mL methanol (Lichrosolv; Merck, Darmstadt, Germany) at 4°C. The extracts were centrifuged at 16,000g (Eppendorf centrifuge 5415; Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) for 5 min, and the supernatant was subsequently dried in a stream of nitrogen. Dried latex extracts were stored at -20°C under argon until further extractions. Plant leaf samples were homogenized in liquid nitrogen. Powdered leaf material (1 g) or dried latex extract (300 mg) was extracted with methanol/water (17:3, v/v) according to the method of Evans and Soper (10). The extract was partitioned against hexane to remove nonpolar substances. The methanol/water ratio was changed to 1:2 and diterpene esters were extracted with diethylether. The recovery of phorbol-12-tetradecanoate-13-acetate (TPA) added to dried latex extract was 90%.

Hydrolysis and quantification of diterpenoids. Diterpene esters were transformed into their parent alcohols by alkaline

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hydrolysis (0.5 M methanolic potassium hydroxide, 20 min, 20°C). This method was adopted from Evans and Kinghorn (15) and from Girin et al. (16). The samples were subsequently neutralized with 0.5 M methanolic hydrochloric acid and fractionated by thin-layer chromatography (TLC) on silica gel 60 (Merck), using *n*-hexane/propan-2-ol (2:1,v/v) as a solvent system. Ingenol and phorbol (both from Sigma; Deisenhofen, Germany) and 12-deoxyphorbol, produced by hydrolysis of 12-deoxyphorbol-13-tetradecanoate (Sigma), were used as standards for TLC (Rf values: phorbol, 0.28; ingenol, 0.47; 12-deoxyphorbol, 0.68). The corresponding TLC regions were isolated, extracted with methanol, and analyzed by HPLC on an RP 18 column (250 × 4.6 mm, Spherisorb ODS2, 5 µm; Bischoff, Leonberg, Germany) with a water/acetonitrile gradient (flow rate: 1 mL/min; solvent A: 100% H₂O; solvent B: 88% acetonitrile, 12% H₂O; 5 min A, linear gradient to B in 40 min, 10 min B). The compounds were detected by ultraviolet absorption (220 nm). Identification of ingenol and deoxyphorbol was carried out by comparison of retention times of standards and by mass spectroscopy (Finnigan MAT SSQ 7000; Finnigan-Thermoquest, Egelsbach, Germany). The detection limit of ingenol in the HPLC system was at 0.2 nmol/sample.

Cell culture and luciferase assay. Raji cells, which contained a firefly luciferase reporter gene under the control of the EBV-DR promoter in an autoreplicative plasmid, were used as described by Polack et al. (14). Cells were grown at 37°C in a humidified atmosphere with 5% CO₂ in medium RPMI 1640 containing 10% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, 50 µg/mL streptomycin, and 300 µg/mL hygromycin B. The medium and its supplements were supplied by Gibco BRL (Eggenstein, Germany). The cells were treated with 1–20 µL plant extract (or known inducer) for 2 days in 24-well plates. The final volume per assay was 1.5 mL. Control measurements were carried out with plant extracts from non-*Euphorbiaceae* indoor plants (*Ficus benjamina*, *Ficus elastica*, and *Clusia minor*) and with methanol instead of plant extracts. The luciferase assay was carried out according to the instructions supplied with the Promega luciferase assay system (Promega GmbH, Mannheim, Germany). A tube luminometer (Autolumat LB 953; Berthold, Bad Wildbad, Germany) was used. The light response was always in the linear range of the reaction. Relative luciferase activity was calculated on the basis of protein determined by the procedure of Bradford (17).

Results

Analytical detection of diterpenes. Alkaline hydrolysis of unknown diterpene esters in plant extracts to their parent C 20-alcohols, and their subsequent purification by TLC and HPLC, were suitable methods for the quantitative estimation of diterpene esters. In our experimental plants, derivatives of ingenol, but not of phorbol or 12-deoxyphorbol, could be detected. Figure 1 shows typical HPLC chromatograms for an ingenol standard and two different plant extracts. Ingenol was quantified by integration of its peak area.

Several *Euphorbiaceae* indoor-plant cultivars obtained commercially showed a broad range of ingenol ester contents. The analytical data for latex and leaves are presented in Figure 2. In the investigated *E. lomii* hybrids the mean ingenol content was 73 ± 18 ng/mg latex. The Gabi cultivar contained 135 ± 36 ng/mg latex. The diterpenes were located in the milky latex; concentrations in the total leaf extracts were lower than or even below the detection limit. No diterpene esters could be detected in the different cultivars of *E. pulcherrima* (*Poinsettia*). The cultivars of *Co. variegatum* were also deficient in the three toxic diterpenes in the sap (clear fluid, no milky emulsion) and in the total leaf extracts. The highest concentration of

ingenol was detected in the latex of *E. leuconeura* (744 ± 126 ng/mg latex). As negative controls, nonsurge plants (*F. benjamina*, *F. elastica*, and *Cl. minor*) were analyzed and were devoid of diterpene derivatives.

Test for tumor-promoting potential. EBV induction was used as a measure for tumor-promoting potential of the plant extracts. The luciferase reporter gene under the control of an EBV early promoter was introduced into Raji cells. The EBV promoter is induced to high levels only in the presence of EBV-inducing agents (14). Figure 3A shows a dose-response curve for TPA, a well-known standard tumor promoter. Induction occurred at a threshold concentration of 0.7–1 nM TPA. Below this concentration luciferase activity was comparable to controls. Concentrations above threshold induced an up to 200-fold increase in activity. Maximum induction was achieved at 2 nM TPA. A further increase in concentration caused a slight reduction in luciferase induction. However, even at high TPA concentrations, induction was still 50- to 100-fold above control. Because the plant species studied contained only ingenol derivatives, authentic ingenol 3,20-dibenzoate was tested

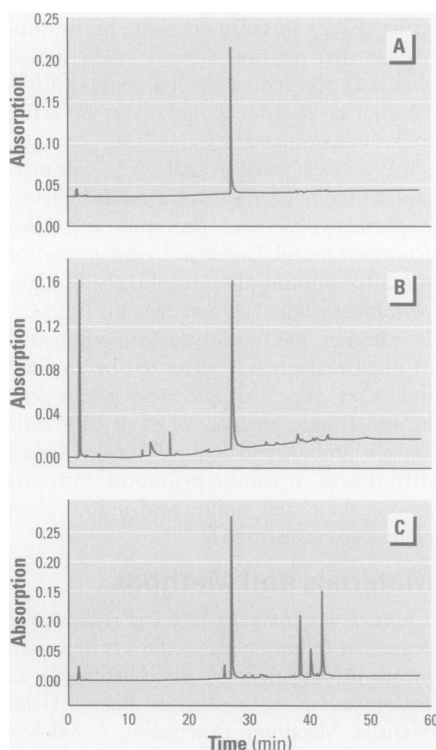


Figure 1. Typical high-performance liquid chromatography chromatograms of (A) an ingenol standard and of the following hydrolyzed latex samples in an acetonitrile/water gradient on an RP 18 column: (B) *Euphorbia lomii* hybrid Gabi and (C) *E. leuconeura*.

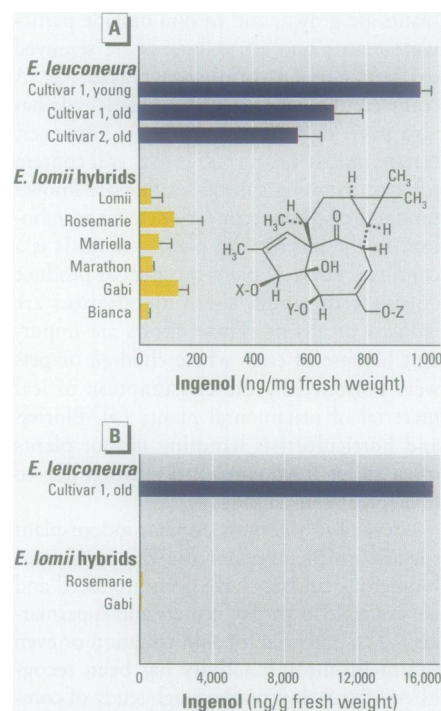


Figure 2. Ingenol contents of spurge indoor-plant cultivars. Concentrations were determined by high-performance liquid chromatography analysis after hydrolysis of the plant extracts as described in "Materials and Methods." (A) Data from latex extracts and the structure of ingenol. X, Y, and Z indicate the location of ester bonds. (B) Corresponding total leaf extracts. Only data from plants with detectable ingenol concentrations are presented. Data represent mean \pm standard error, $n = 3-7$.

as a further standard. A dose-response curve with a threshold concentration of 5–20 nM was obtained (Figure 3A). This threshold concentration was approximately 20-fold higher than that for TPA. The induction maximum was 30 nM. Higher concentrations led to a slight reduction of induction.

Several dilutions of plant latex extracts were tested in the induction assay to examine for a correlation with ingenol ester concentration (Figure 3B). Equal ingenol contents of the latex extracts of *E. lomii* Gabi and *E. leuconeura* produced identical induction curves. The samples were isolated from two plant species, but their potential to activate EBV promoter was the same. The threshold concentration was approximately 1 nM. The induction maximum was 5 nM. The induction curve was therefore comparable to those of the tested tumor promoter standard TPA. The identical dose-dependent inductions by two plant extracts pointed to a direct correlation between ingenol content and promoter activity. This conclusion was supported by HPLC fractionation of latex extracts as shown in Figure 4 for *E. lomii* Gabi. Only fractions that contained ingenol derivatives

exhibited EBV induction. In addition to latex extracts, the corresponding total leaf extracts were tested for EBV induction. *E. leuconeura* and *E. lomii* Gabi leaf extracts were highly active.

The popular indoor plant *Co. variegatum* is a representative of the *Euphorbiaceae* that does not contain a milky latex. The transparent exudate from cultivar Batic Red isolated after scalpel incisions failed to activate the EBV promoter. The latex from non-*Euphorbiaceae* control plants (*F. benjamina*, *F. elastica*, and *Cl. minor*) likewise was devoid of EBV-inducing activity. However, with high amounts of undiluted control extracts a slight induction was observed. Such nonspecific activation also had to be considered for latex extracts of *E. pulcherrima* that released no ingenol after hydrolysis. These latex extracts led to a low EBV induction only when the applied extracts were increased 500–5,000-fold relative to *E. leuconeura* (data not shown).

Discussion

Analytical aspects. Members of the spurge family rank among the commercially most important indoor plants in Germany and in other countries. There is some uncertainty with regard to the potential danger deriving from these plants (5). Avoidance of skin contact and of oral uptake has been recommended (8). In our experiments, a total of 22 cultivars of commercial indoor plants of the spurge family were analyzed. When latex and leaf extracts were examined by HPLC, no ingenol could be found in seven cultivars of *E. pulcherrima* and in eight cultivars of *Co. variegatum*. Cultivars of *E. lomii* hybrids and in particular of *E. leuconeura* contained ingenol derivatives in latex. Phorbol- and 12-deoxyphorbol esters were absent. The latex of *E. lomii* showed ingenol concentrations between 30 and 135 ng/mg latex, depending

on the tested cultivar. *E. leuconeura* is a rare indoor plant that is often mistaken for the related *E. lophogona*. The latex contained up to 1 µg ingenol per milligram latex. The ingenol esters of *E. leuconeura* have recently been shown to belong to the milliamine-type of diterpene esters (18,19). These compounds show a close relationship to those of the more popular *E. milii* (20–22).

Because many EBV inducers also act as tumor promoters, the present EBV-induction assay is generally accepted to indicate a tumor-promoting potential of chemical substances (13). Latex and leaf samples of *E. lomii* hybrids and *E. leuconeura* were strong inducers. There was a close correlation between EBV induction and ingenol content. With *E. pulcherrima*, an induction could only be observed when unreasonably high amounts of extract were applied. In this range a nonspecific induction also occurred with non-*Euphorbiaceae* control plants, namely two species of *Ficus* and *Clusia minor*.

Environmental aspects. Many of the wild spurge plants at their natural stands contain high amounts of diterpene esters with tumor-promoting potential (23). The most famous representative of this group is TPA. The present study reveals that the most popular cultivars of *Euphorbiaceae* indoor plants (*E. pulcherrima*, *Co. variegatum*) are devoid of tumor promoters. Prolonged cultivation and selection has apparently also led to low levels of tumor promoters in the various *E. lomii* hybrids. However, an element of risk for collectors and lovers of rare *Euphorbiaceae* indoor plants and to the gardeners producing them was detected in *E. leuconeura*. Apparently, rare commercial *Euphorbiaceae* indoor plants are produced from the wild plant species in their native stands. The ingenol concentration found in the latex must be regarded in relation to the volume of latex that results from an injury of the leaves or the stem. *E. leuconeura* showed significant amounts of latex after small scalpel incisions were made in the leaves or stems. The varieties of *E. lomii* hybrids in our experiments showed release of much less latex. In several varieties such as Bianca and Rosemarie it was difficult to collect latex at all. Leaf-surface wiping tests of *E. leuconeura* failed to liberate ingenol derivatives. In summary, the HPLC and bioassay methods presented in this study could provide a basis for the development of cultivars free of tumor-promoting metabolites and perhaps for consumer-oriented labeling.

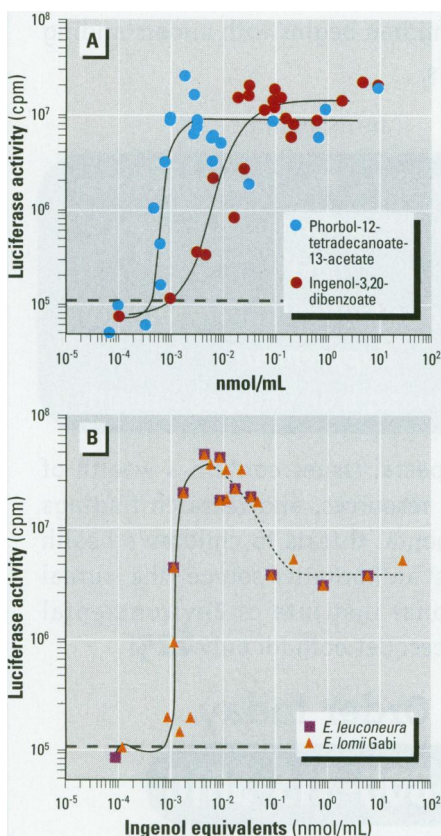


Figure 3. Epstein-Barr virus-inducing activity. (A) Phorbol-12-tetradecanoate-13-acetate and ingenol-3,20-dibenzoate standards. (B) Latex extracts of *Euphorbia leuconeura* and of *E. lomii* Gabi. Ingenol content was determined by hydrolytic treatment of the extracts. The dashed lines represent control values without induction.

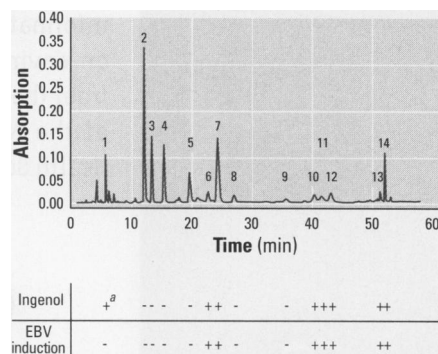


Figure 4. Fractionation of *Euphorbia lomii* Gabi latex extract by high-performance liquid chromatography. EBV, Epstein-Barr virus. Parallel tests for ingenol compounds and for EBV-inducing activity are shown.

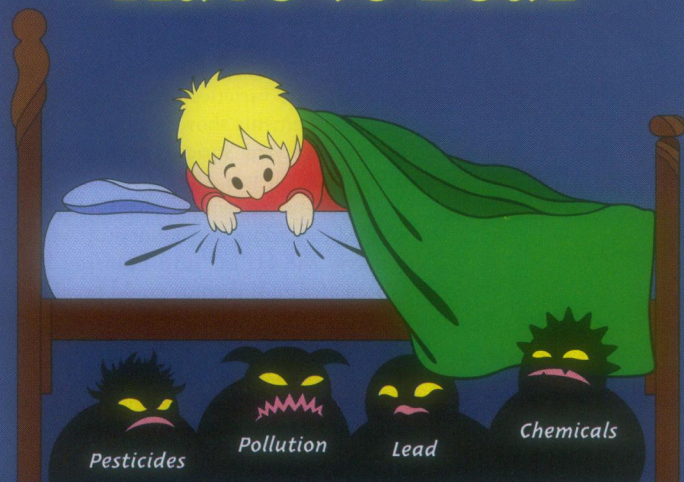
*Free ingenol probably due to degradation of esterified compounds during storage.

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